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Research Article

A study on Probiotic potential of Lactic acid bacteria (LAB) and evaluating their effectiveness against enteric and fungal infections.

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Abstract

Lactic acid bacteria and yeast are well known as probiotics and bio-preservative. They have ability to protect the gastrointestinal tract from urogenital infections and other harmful pathogen by maintaining the acidic condition. In the present study Lactic acid bacteria and yeast were isolated from milk and curd. The isolates were tested for probiotic activity and then were screened for bacteriocin production. Bacteriocin like protein was partially purified by ammonium sulphate precipitation method and the purified protein was used for antimicrobial activity screening against both bacterial and fungal pathogens.

Introduction

Probiotics are dietary supplements which enhance the growth and health of the host animals. Therefore, they have received a lot of attention as a biological means of disease control, digestion aids, immune booster and supplementing or replacing the use of synthetic antimicrobial compounds in the field of health and medicine (Chantharasophon et al., 2011, Heller,2001, O'Mahony et al., 2005). Various types of microbial strains have been employed for probiotic production. Among these are *Lactobacillus* and *Bifidobacterium* (Critchfieldet al., 2011), which have not shown any risk to humans which was clearly demonstrated by Saxelin et al., (1996) and Naidu et al., (1999).

Organic acids such as lactic acid and acetic acid produced by lactic acid bacteria are important antimicrobial compounds, and have been reported to possess antifungal activity (Gerez 2009). Among probiotics, the case of *Saccharomyces cerevisiae* is interesting to consider, since yeast has been used for decades, as both preventive and therapeutic agent for diarrhea and other gastro-intestinal disturbances in humans. Todorov and Dicks, 2006 and Klare *et al*., 2007 have reported that probiotic strains isolated from animal and human GI tract show antibiotic resistance. Bacteriocin like substances are also produced by lactic acid bacteria but have relatively narrow inhibitory spectra. In general, they tend to be against wide range of gram positive, and some have also been reported to inhibit gram negative species. Milani *et al*. (1998) reported that the strains of the lactic acid bacteria are one of the most relevant biocontrol agents and an alternative to chemical medicines (Janiswicz & Bors, 1995).

Thus , the present study involved the isolation of endemic strains of LAB and consequentl;y screening them for their probiotic and antifungal and antibacterial activity which was a result of production of Bacteriocin like proteins by the isolated strains.

Materials and Methods

Sample

For isolation of Lactobacilli strains cow milk, buffalo milk, goat milk and curd were used. These were collected in sterile bottles.

Isolation of *Lactobacillus*

MRS (de Man Rogosa and Sharpe) medium were used for the isolation of *Lactobacillus sp.* Dilutions of the various types of milk was done in sterile water and then they were spread

plated on MRS agar plates. The plates were inoculated at both 37 ^{0}C and 25 ^{0} C for 24 to 48 hours. Distinctive colonies having the characteristic appearance as large, white colonies were picked up and studied further.

Identification of isolates

The isolates were identified on the basis of Bergey's Manual of Systematic Bacteriology (Holt *et al*., 1984) and ABIS encyclopedia**.** The isolated strains were differentiated on basis of colony characteristics like colour, texture and size of colony and Gram stain. Different Biochemical test were conducted and the lactobacilli strains were identified. The various biochemical test performed were: IMViC, Catalase, Sugar fermentation tests (glucose, fructose, galactose, maltose, mellibiose, lactose, sucrose, raffinose, mannose, arabinose, ribose, amygdalin, adonitol, arabitol, cellobiose, fucose, gluconate, glycogen, glycerol, inositol, trehalose etc) , Gelatin utilization, Casein utilization, Starch Utilization, Urease Test, H_2S production, Nitrate reduction, oxidase test, etc.

Probiotic Activity Test

Survival in gastro intestinal tract

Survival of Lactobacillus in the gastrointestinal tract or gut is important for its probiotic action. The lactobacillus should survive as well as adhere to the mucous lining of the gut walls in order to prevent the binding of pathogenic and harmful bacterial cells . *Lactobacillus* spp. isolated were inoculated on MRS agar plated having different pH 2, 3, 4, 5, 6 and 7.

Milk technological properties

Lactobacillus has a very strong property of causing the curdling of milk. For this, fresh milk was taken in 6 test tubes and one test tube was marked as control. All the 5 test tubes were inoculated with the 5 isolated strains of *Lactobacillus* and kept in incubator for 24hrs at 37^{o}C. Change of milk into curd by the *Lactobacilli* strain was considered as a positive indicator.

Effect of different salt concentration on bacterial isolates

The other physiological parameter for growth of a cell is the requirement of sodium chloride, as the physiological saline prevents the cell from osmotic shock. Various concentrations of sodium chloride (NaCl %) i.e. 2%, 4% and 6% were used to observe the effect of bacterial isolates at different salt concentrations. The bacterial strains were streaked in the MRS Agar plates and kept in incubator at 37^{o}C for 24hrs.

Antibacterial activity

The effectivity of the Bacteriocin produced and the combination of lactic acid and probiotic effect was checked by Agar well diffusion method. The different isolated bacterial strains such as *E. coli, Staphylococcus aureus, Enterobactor aerogenes, ,Pseudomonas aurogenosa, Bacillus subtilis, Paenibacillus and Clostridium sp.* were taken and swabbed with sterile cotton over the entire surface of the pre-set solidified NA petri plate. Two wells were already made before bacterial swabbing, of which one well was filled with 100µl of bacteriocin of isolated strains and second was filled with 100µl of sterile distilled water and the plates were incubated for 24 hrs at 37°C and zone of inhibition was observed.

Antifungal activity

The fungal strains were isolated from soil, waste water, infected leaf of tomato and hair dandruff and were inoculated in PDA media.Antifungal activity was also done by above mentioned agar well diffusion method. The different fungal strains such as *Aspergillus niger, Aspergillus flavus, Alternaria alternata, Penicillium citrinum and malassezia furfur* were taken for testing of antifungal activity. The PDA media were poured on petri plate. Two wells were made in which one well was filled with 100µl bacteriocin and other with 100µl sterile distilled water.

Antibiotic resistance

100µl of each different antibiotic were mixed in MRS Agar media (25ml)and was poured on petriplate and after that different lactobacillus and yeast culture were swabbed on MRS Agar plates. Then these plates were incubated at 37^{0}C for 24 hrs. The antibiotics resistance activity was also studied by the disc diffusion methods.

Extraction of Bacteriocin from LAB cultures

A loopful of each of the different *Lactobacillus* culture and yeast culture were inoculated in freshly prepared MRS broth and were incubated for 48 hrs.at 37^{0}C. After that the cultured broths were centrifuged at 5000 rpm for 15 min. The supernatant were taken in new tubes. This was the crude bacteriocin of LAB and yeast.

Partial purification of bacteriocin

The isolated strains were cultivated on MRS broth at 37^{0}C for 48 hrs. After incubation, the broth was centrifuged at 5000 rpm for 15 minutes and the cells were separated out. This supernatant was used as crude bacteriocin. The ammonium sulphate was added to the supernatant by stirring on the magnetic stirrer. Adding of ammonium sulphate were done until the solubility of it stopped in supernatant and the particles of ammonium sulphate were seen at the bottom of

crude bacteriocin then it was kept undisturbed at 4^0C overnight. The precipitates formed were collected by centrifugation at 10000 rpm for 15 minutes and redissolved in 20 mM sodium phosphate buffer of pH 6.5. This was the purified bacteriocin like proteins

Antimicrobial activity of partially purified probiotcs bacteriocin

Partially purified proteins were tested against different bacterial and fungal pathogens by agar well diffusion method.

Results and Discussion

Isolation and identification of probiotic strains

The probiotic strains were isolated from milk and curd. Gram staining showed 4 strains to be gram positive, rod-shaped and additionally yeast cells were also obtained on MRS media itself. Yeast was maintained on Potato dextrose agar and the other strains were maintained on MRS agar plates.

Biochemical tests

Comparison of the biochemical test results showed clearly that Lac B strain was found similar to *Lactobacillus viridens* , Lac C strain was found similar to *Lactobacillus helviticus* , Lac F strain was found similar to *Lactobacillus planatarum* , Lac A strain was found similar to *Bacillus cereus*. The test results are given in Table no. 1.

Table No: 1 Biochemical test results for the identification of different cultures

Probiotic Strains	Lac B	Lac C	Lac F	Lac A
Biochem. tests				
Indole	-	-	-	-
Methyl red	-	-		-
Voges prosker	-	-	-	-
Citrate	-	-	-	-
Glucose	-	+	+	-
Sucrose	-	-	+	-
Fructose	+	-	+	-
Maltose	+	+	+	-
Lactose	+	+	+	-
Mannitol	-	-	+	-
Galactose	-	+	+	+
mellibiose	-	-	+	+
raffinose	-	-	+	+
mannose	-	+	+	+
arabinose	+	-	+	+
ribose	+	+	-	+
cellobiose	+	+	+	-
inositol	+	-	-	-
glycerol	+	-	-	-
Catalase	-	-	+	+
H2S	-	-	-	-
Casein hydrolysis	+	+	+	+
Urease	-	-	-	-
Gelatinase	+	+	+	+
Starch Hydrolysis	+	--	--	--
Ammonia producn.	+	+	+	+
Oxidase	+	+	+	+
Glycoprotein	+	+	+	+

Survival in gastro intestinal tract

The probiotic strains of Lactobacillus and yeast showed growth at 3, 4, 7, 8 pH. But at pH 8 *L. helveticus* and *Bacillus cereus* showed poor growth and at pH 3, yeast shows poor growth. This shows that the LAB strains isolated were having good capability to survive the environment in the gastro intestinal tract as shown in table No: 2.

Table No. 2: Survival o different LAB isolates on low pH.

Probiotic strains	pH 3	pH 4	pH 5	pH 6	pH 7	pH 8
Lac B*(L.viridens)*	+	++	+	+	+	-
Lac C*(L.helviticus)*	++	++	+	+	+	-
Lac F(*L.planatarum)*	++	++	+	+	+	+
Lac A*(B . cereus)*	+	+	+	+	++	+
S. cerevicae	-	+	++	++	++	+

Milk technological properties

Lactobacillus has a very strong property of causing the curdling of milk. Change of milk into curd by the *Lactobacilli* strain was considered as a positive indicator. All the five isolates showed very good curdling of milk in comparison to the control. This indicates that all the LAB isolates were producing organic acid which resulted in lowering of pH and thus curdling.

Effect of different salt concentration on bacterial isolates

Various concentrations of sodium chloride (NaCl %) i.e. 2%, 4% and 6% were used to observe the effect of bacterial isolates at different salt concentrations. The bacterial strains were streaked in the MRS Agar plates and kept in incubator at 37^{o}C for 24hrs. All the bacteria isolated showed growth even at 6% NaCl concentration which indicates that the LAB strains would survive in the intestine having large amount of digested food and antibiotics and drugs. These would also help in better absorption and assimilation of nutrition.

Antibacterial activity

The antimicrobial activity of LAB and *S. cerevicae* and their zone of inhibition against various test pathogens were studied. The supernatant of probiotics showed zone of inhibition when tested against the indicated strains of bacteria and fungi.

Table 3 : Zone of Inhibition (in mm) against pathogenic Bacterial cultures.

Probiotic strains	***E. coli***	***Enterobactor cloacae***	***Staphylo coccus aureus***	***Bacillus subtilis***	***Pseudomonas aeruginosa***	***Paeni bacillus sp.***	***Clostridium sp.***
Lac B *(L.viridens)*	25	21 mm	16 mm	22 mm	20 mm	22 mm	20 mm
Lac C *(L.helviticus*)	13 mm	7 mm	10 mm	6 mm	15 mm	20 mm	21 mm
Lac F *(L.planatarum)*	13 mm	8 mm	6 mm	4 mm	14mm	21 mm	22 mm
Lac A *(B . cereus)*	14 mm	22 mm	10 mm	13 mm	14 mm	15 mm	21 mm
S. cerevicae	_	_	22 mm	21 mm	13mm	14 mm	20 mm

The results of the antibacterial activity show that *L.viridens* showed higher zone of inhibition against all the bacterial strains. It was active against both gram + ve as well as gram –ve bacterial strains.The other lactobacilli strains showed better inhibitory activity against gram +ve bacteria to a higher level as compared to gram –ve bacteria.

Antifungal activity:. The different pathogenic fungal strains such as *Aspergillus niger, Aspergillus flavus, Alternaria alternata, Penicillium citrinum and malassezia furfur* (dandruff causing fungi) were taken for testing of antifungal activity. The surprising results showed that *L.viridens and* yeast had the maximum antifungal activity against all the fungal pathogens. While all other LABs isolated showed good antifungal activity against all fungi except for *Pencillium sp.*

Table 4 : Zone of inhibition showed against different fungal pathogens.

Probiotic strains	*Aspergillus niger*	*Aspergillus flavus*	*Malassezia furfur*	*Pennicillium sp.*	*Alternaria alternata*
Lac B *(L.viridens)*	24 mm	20 mm	24 mm	25 mm	24 mm
Lac C *(L.helviticus*	22 mm	23 mm	15 mm	12 mm	20 mm
Lac F *(L.planatarum)*	23 mm	24 mm	14 mm	13 mm	24 mm
Lac A *(B. cereus)*	23 mm	24 mm	20 mm	12 mm	20 mm
S. cerevicae	30 mm	32 mm	20 mm	25 mm	26 mm

Antibiotic resistance

The LAB and yeast culture showed resistance against various types of antibiotics such as Ampicillin, Tetracycline, Streptomycin, Penicillin, Neomycin, Leofloxin, Gentamycin, Rifamycin, Cefuroxime, Angefix, Novamox etc. The result is shown in Table no. 5.As depicted all the strains were resistant to majority of the antibiotics. Thus, these LAB can be used as supplement so that the natural flora and fauna of the gastrointestinal tract can be maintained.

Table 5: Antibiotics Sensitivity and Resistance of probiotic strains.

Antibiotics / Probiotic strains	Ampi-cillin	Tetra-cyclin	Strepto-Mycin	Penici llin	Neo-mycin	Leofl - Oxin	Genta - mycin	Rifa-mycin	Cefuro-xime	Ange - fix	Nova-mox
LacB (*L.viridens)*	+	+	+	+	+	+	_	+	+	+	+
LacC (*L.helviticus)*	+	+	+	+	+	+	_	+	+	+	_
LacF (*L.planatarum)*	+	+	+	+	+	+	_	+	+	+	_
LacA (*B . cereus)*	+	+	+	_	_	+	_	+	+	+	+
S. cerevicae	+	+	+	+	+	+	+	+	+	+	+

Extraction and Partial purification of Bacteriocin

A loopful of each of the different *Lactobacillus* culture and yeast culture were inoculated in freshly prepared MRS broth and were incubated for 48 hrs.at 37^0C. After that the cultured broths were centrifuged at 5000 rpm for 15 min. The supernatant was used as the crude bacteriocin like protein of LAB and yeast. After purification of the bacteriocin like protein it was screened for antimicrobial activity

Protein Quantification

The protein crude and purified were quantified.

Table 6 : Protein quantity (mg/ml) after 24 hr. 72 hr.

Probiotic strains	Protein mg/ml after 24 hr.	Protein mg/ml after 72 hr.
Lac B*(L.viridens)*	1.27	0.72
Lac C*(L.helviticus)*	0.72	0.90
Lac F (*L.planatarum)*	0.59	0.77
Lac A*(B . cereus)*	0.81	1.00
Yeast	0.42	0.54

Antimicrobial activity of partially purified bacteriocin:

Table 7. Zone of inhibition (in mm) of the probiotic strains against different bacteria.

Probiotic strains	*E. coli*	*Enterobactor aerogenes*	*Staphylo- coccus aureus*	*Bacillus subtilis*	*Pseudomona saeruginosa*	*Paenibacillu ssp.*
L. viridens	28 mm	18 mm	23mm	26 mm	19 mm	18 mm
L. helveticus	18mm	21 mm	18 mm	20 mm	21mm	21 mm
L. plantarum	21 mm	15 mm	20 mm	24mm	22mm	18 mm
B. cereus	22 mm	20 mm	21mm	25 mm	18 mm	16 mm

Here, results of the present study show that purification of bacteriocin like proteins lead to increase in the antimicrobial activity. This confirms the fact that the antimicrobial activity of LABs is due to the bcateriocin like protein and also to some extent the Lactic acid produced during growth.

The possible mechanism of bacteriocin resistance of gram negative and some gram positive bacteria has been suggested to be associated with the barrier properties of the outer membrane and cell wall. According to observations of Jack, *et al (*1995) antibiotics form defined pores in membranes, rather than producing the generalized membrane disruption. Confirmation of the membrane deenergizing action of the lantibiotics has been achieved by direct measurement of the membrane potential or lantibiotics treated cell. The non lanthionine containing bacteriocins also appear to affect their bacterial action by destabilizing the cytoplasmic membrane of sensitive cells; however, the mechanism through which they achieve this appears to differ somewhat from that described for the lantibiotics. Aldunateet al., (2013) reported that L-lactic acid at 0.3% (w/w) was 17-fold more potent than D-lactic acid in inactivating HIV_{Ba-L}. Complete inactivation of different HIV-1 subtypes and HIV-2 was achieved with ≥0.4% (w/w) L-lactic acid.

It was summarized from the conducted study that isolate used in this study was found as gram positive *Bacilli* and *yeast* showing oval shaped morphology. The above result of probiotic strains, it was found that the probiotic strains shows good effective antimicrobial activity on different microbes *Bacillus, Staphylococcus, E. coli, Closridium, Enterobacter, Penibacillus, Pseudomonas, Alternaria alternata, A.niger, A.flavus, Malassezia, and Penicillium*,but S. cerevisae does not shows any zone of inhibition against *E. coli* and *Enterobacter.* Inhibitory activity of *Saccharomyces cerevisiae* on the adhesion of *Entamoeba histolytica* trophozoites (Rigothier*et al.*, 1994), *Staphylococcus aureus* (Elliot *et al.*, 1991) to human cells, *Salmonella typhimurium* and *Shiguella flexneri* (Rodrigues et al. 1996) in mice has also been shown. The probiotic strains shows resistance to above different antibiotics, and shows sensitivity that is *L.viridens* against gentamycin, *L. helveticus* against Gentamycin (GM),Novamox, Levofloxacin (QB), Linezolid (LZ), Ciprofloxacin (RC), Cefotaxime (CF), Cephalexin(PR) sensitive for Gentamycin and Novamox sensitive*B. cereus* against Gentamycin, Penicillin, Neomycin,Cefotaxime(CF), Cephalexin (PR) sensitive. The probiotic strains changed milk into curd and also shows growth on different pH3-8. The partially probiotic proteins also represent the zone of inhibition and conformed the antimicrobial activity. Therefore according to the present study, more research, especially in the form of well-designed clinical trials, is needed to evaluate the efficacy and safety of probiotics. With evolving knowledge, effective probiotic therapywill be possible in the near future.

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